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Comparison of five commercial DNA extraction kits for the recovery of *Francisella tularensis* DNA from spiked soil samples

Chris A. Whitehouse[a,*], Hannah E. Hottel[b]

[a]*Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA*
[b]*Oak Ridge Institute for Science and Education (ORISE) United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA*

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Abstract

Francisella tularensis is the etiologic agent of the zoonotic disease tularemia and is thought to be maintained in the environment principally by various terrestrial and aquatic vertebrate animals. The organism is known to persist in water or mud for long periods of time and *Francisella*-specific DNA has been identified from water and soil. To gain a better understanding of the ecology and epidemiology of *F. tularensis*, it will be important to further explore its distribution in the environment. Therefore, methods must be established to efficiently extract *Francisella*-specific DNA from the soil and be able to eliminate potential PCR inhibitors. Thus, we evaluated five commercial DNA extraction kits for their ability to recover *F. tularensis*-specific DNA from soil samples and eliminate potential PCR inhibitors. The kits evaluated included the Puregene DNA purification kit, QIAamp Stool Mini kit, Epicentre Biotech SoilMaster DNA extraction kit, and the UltraClean™ and PowerMax™ soil DNA isolation kits from MoBio. Soil samples were spiked with γ-irradiated *F. tularensis* SHU-4 strain (corresponding to a range from 10 to 10^5 CFU). Spiked samples were extracted with each kit and evaluated using a *F. tularensis*-specific real-time PCR assay and an internal positive control assay that measures the presence of potential PCR inhibitors. DNA extraction using the UltraClean™ and PowerMax™ kits resulted in the most consistently positive results at the lowest limit of detection (20 and 100 CFU/g soil, respectively) for all soil types tested, suggesting that these kits can provide the most sensitive methods for extracting *F. tularensis* from environmental soil samples. Processing time and cost were also evaluated. Published by Elsevier Ltd.

Keywords: *Francisella tularensis*; Soil extraction; Molecular diagnostics; Bioterrorism

1. Introduction

Francisella tularensis is a gram-negative bacterium and the etiological agent of tularemia, a zoonotic disease of humans, which is widespread throughout the Northern Hemisphere [1–3]. *F. tularensis* is highly infectious; exposure to less than 10 organisms is able to cause disease. The highly infectious nature of the organism has made it attractive as a bioweapon in historical biological warfare research programs and has prompted the US centers for disease control and prevention (CDC) to list it as a Category A bioterrorism agent [4]. The species *F. tularensis* is currently considered to have four subspecies: *tularensis*, *holartica*, *mediasiatica* and *novicida* [3]. *F. tularensis* subsp. *tularensis*, also known as Type A, tends to be more virulent both for animals and humans. *F. tularensis* subsp. *holartica* (formerly described as *paleartica*) and known as Type B is more common in Europe and Asia, and tends to cause a less virulent form of disease than subspecies *tularensis*. The live vaccine strain (LVS), used as a live-attenuated vaccine product, is a strain in this subspecies. *F. tularensis* subsp. *mediasiatica* has only been recovered sporadically from ticks and animals in regions of Central Asia. *F. tularensis* subsp. *novicida* was first recovered from water in Utah, USA in 1951 and has rarely caused human disease; however, it was isolated for the first time in the Southern Hemisphere from a foot wound infection sustained in brackish water in the Northern Territory of Australia [5].

*Corresponding author. Tel.: +301 619 2098; fax: +301 619 2492.
E-mail address: chris.whitehouse@amedd.army.mil (C.A. Whitehouse).

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14. ABSTRACT

Francisella tularensis is the etiologic agent of the zoonotic disease tularemia and is thought to be maintained in the environment principally by various terrestrial and aquatic vertebrate animals. The organism is known to persist in water or mud for long periods of time and Francisella-specific DNA has been identified from water and soil. To gain a better understanding of the ecology and epidemiology of F. tularensis, it will be important to further explore its distribution in the environment. Therefore, methods must be established to efficiently extract Francisella-specific DNA from the soil and be able to eliminate potential PCR inhibitors. Thus, we evaluated five commercial DNA extraction kits for their ability to recover F. tularensis-specific DNA from soil samples and eliminate potential PCR inhibitors. The kits evaluated included the Puregene DNA purification kit, QIAamp Stool Mini kit, Epicentre Biotech SoilMaster DNA extraction kit, and the UltraClean and PowerMax soil DNA isolation kits from MoBio. Soil samples were spiked with gamma-irradiated F. tularensis SHU-4 strain (corresponding to a range from 10 to 10(5)CFU). Spiked samples were extracted with each kit and evaluated using a F. tularensis-specific real-time PCR assay and an internal positive control assay that measures the presence of potential PCR inhibitors. DNA extraction using the UltraClean and PowerMax kits resulted in the most consistently positive results at the lowest limit of detection (20 and 100CFU/g soil, respectively) for all soil types tested, suggesting that these kits can provide the most sensitive methods for extracting F. tularensis from environmental soil samples. Processing time and cost were also evaluated.

15. SUBJECT TERMS

methods, DNA extraction, Francisella tularensis, kit comparison, spiked soil samples

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Rodents, hares, and rabbits are important sources of human infection; however, they may not represent true reservoirs because these species develop acute infections. Alternatively, it has been suggested that the bacterium might be able to survive long periods in infected ticks. An alternative suggestion is that the bacterium is able to persist free-living in the environment or in protozoa. The fact that *F. tularensis* has been shown to survive in amoebae in the laboratory [8], and has been isolated from natural water sources [9,10], seems to support this idea. In addition, it has been shown that *F. tularensis* can persist in a viable but nonculturable form in water [11], which may play a role in the maintenance of this bacterium in the environment. *Francisella* strains are extremely difficult to culture from environmental sources [12]; however, recent molecular surveys have identified *Francisella*-like bacteria in soil samples from Houston, TX, Denver, Co [13], and from Martha's Vineyard, MA [14], suggesting new groups of diverse *Francisella* may be widespread in the environment.

A large number of methods have been published for extracting DNA from soil [15–22]. However, these methods include a variety of procedures such as phenol/chloroform extraction, cesium chloride density gradient centrifugation, and column chromatography, which are laborious and time-consuming and not suited for processing large numbers of samples. Furthermore, many of the previously published studies did not evaluate their methods for removing PCR inhibitors or have likewise used laborious methods such as chemical flocculation [23] or column chromatography with a variety of gel filtration resins [24] for the removing PCR inhibitors.

The objectives of this study were to compare five commercial DNA extraction kits for their ability to specifically extract *F. tularensis* DNA from a variety of soil types. The efficiency of extraction was evaluated using a *F. tularensis*-specific real-time PCR assay, which targets the *fopA* gene [25]. PCR inhibition was measured with a real-time PCR inhibitor assay [26]. The results of this study will prove valuable for future work examining the environmental sources of *F. tularensis* or other environmental pathogens.

2. Materials and methods

2.1. Soils

Three soil types were used to evaluate the efficiency of DNA extractions and purification procedures and included silt loam, clay, and a commercial potting soil. The silt loam soil was collected from a riverbed of the Monocacy River, Frederick County, MD The clay soil was collected from a cornfield in Carroll County, MD, and the potting soil was purchased from Fafard, Inc. (Agawam, MA). All soils were stored at 4 °C until used. The percent moisture content was determined after cooking 10 g of each soil type in a microwave set on high for 30 s intervals. At each interval, weights were recorded, and the process was repeated until no additional change in weight was observed. Particle size distribution, organic content, pH, and elemental analysis were performed by a commercial agricultural testing laboratory (A&L Eastern Laboratories, Inc., Richmond, VA).

2.2. Bacteria and soil spiking

F. tularensis Shu-4 strain obtained from the Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Diseases (USAMRIID) culture collection was γ-irradiated at 21 kGy and used to spike the soil. Aliquots of soil ranging from 0.1 to 10 g (depending on the extraction kit used) were spiked with 100 µl of *F. tularensis* culture corresponding to a range from 10 to 10^5 CFU.

2.3. DNA extractions

DNA extractions were performed using either the Puregene DNA purification kit, QIAamp DNA Stool Mini kit, Epicentre SoilMaster™ DNA extraction kit, MoBio UltraClean™ kit, or the MoBio PowerMax™ soil DNA isolation kit according to the manufacturers' instructions. All extracted DNA was stored at −20 °C until use.

2.4. Real-time PCR

To determine the recovery of *F. tularensis* DNA from spiked soil samples, a real-time PCR assay specific for the *F. tularensis fopA* gene was carried out as previously described [25] with the LightCycler® (Roche, Indianapolis, IN). Briefly, reactions were carried out in 20-µl volumes. Each assay contained 1X PCR buffer (50 mM Tris pH 8.3; 250 µg/ml of bovine serum albumin [BSA]) (Idaho Technology, Inc.) and 0.2 mM dNTP mix (Idaho Technology, Inc.). Eight tenths (0.8) Unit of Platinum® *Taq* DNA polymerase (Invitrogen) was added per reaction. Five microliters of control/template DNA was added just before analysis on the instrument. Thermal cycling conditions were: 1 cycle at 95 °C for 2 min; 45 cycles of 95 °C for 1 s, and 60 °C for 20 s. Fluorescence was read after each 60 °C step. Data analysis was performed using the LightCycler® software version 4.0. The software uses the 2nd derivative maximum algorithm to calculate the crossing point (Cp), which is the point where the sample's fluorescence curve turns sharply upward corresponding to the first maximum of the second derivative of the curve. An internal positive control (IPC) real-time PCR assay was used to determine the efficiency of the evaluated extraction kits to remove PCR inhibitors. This assay was developed at USAMRIID by using a modification of a previously optimized Taqman® PCR assay targeting the protective antigen gene of *Bacillus anthracis* [26]. The assay is sensitive to a variety of inhibitors, including soil, peat, and leonardite humic acids, and fulvic acid [26].

3. Results

3.1. Soil analysis

Soil types used included silt loam, clay, and a commercial potting soil, with organic matter ranging from 1.8 (silt loam) to 64.2% (potting soil). The silt loam and potting soil were more acidic (pH 6.3) than the clay (pH 7.6). The potting soil had the highest moisture content (69.2%), and the clay had the lowest (6.3%). The moisture content of the silt loam was 11.4%.

3.2. Comparison of DNA extraction methods

The sensitivity of *F. tularensis fopA* detection by real-time PCR after extraction by each of the five commercial DNA extraction kits was compared. Table 2 shows the average Cp values for the *F. tularensis fopA* assay for all five kits with three soil types. At the higher concentration of bacterial spike (10^5), the UltraClean™ kit yielded the lowest Cp values (most sensitive) for all soil types tested. For the potting soil, the Purgene and Epicentre kits were comparable and yielded the next lowest Cp values (23.63 and 23.88, respectively). At the lowest concentration of bacterial spike detected (10 organisms), the Epicentre kit yielded a positive PCR result from the silt loam and the UltraClean™ kit yielded positive PCR results for all the soil types. None of the other kits yielded positive PCR results for any of the soil types at this lowest concentration (Table 1).

To take into account the different amounts of soil extracted with each kit, limit of detections (LODs) were calculated on a per gram basis (Table 2). The UltraClean™ outperformed the other methods, yielding LODs of 20 CFU/g for all soil types. The PowerMax™ kit yielded the next lowest LOD (100 CFU/g) for all soil types.

Each DNA extraction kit was evaluated for its ability to remove PCR inhibitors for each soil type by running 6 replicates of the IPC assay for each extracted DNA sample. All five kits were able to remove PCR inhibitors from the silt loam and clay soil types. For the potting soil extracted with the QIAamp kit, there were PCR inhibitors remaining in 3/6 DNA samples (data not shown).

3.3. Time and cost analysis

The amount of time required for sample processing and cost per sample for each DNA extraction kit was determined. The time required to complete each extraction with the QIAamp, Epicentre, and UltraClean™ methods were $\leqslant$1 h. The PowerMax™ required slightly more time to complete, and the Purgene method required the most time to complete (2 h, 45 min).

The other point to be considered when selecting an extraction method is cost. The cost per sample for the QIAamp, Epicentre, and UltraClean™ kits were comparable. The Purgene kit was the least expensive ($1.00), and the PowerMax™ was the most expensive ($17.50), but it must be kept in mind that this kit is designed for large-scale sample processing. On a per gram basis, the cost of the PowerMax™ was comparable to the other methods ($1.75). Although the Purgene kit was the least expensive ($1.00 per sample), it required the most time to complete (2 h, 45 min) and yielded less than optimal LODs of

Table 1
Efficiency of DNA purification procedures as evaluated by a *F. tularensis fopA* real-time PCR on the Roche LightCycler®

Extraction method	Soil type	Ave. Cp (mean±SD) with the following dilutions of *F. tularensis* spiked into soil					
		10^5	10^4	10^3	10^2	10	PCR Efficiencies[a]
PowerMax™	Silt loam	25.82 (±0.04)	29.80 (±0.19)	33.32 (±0.57)	ND[b]	ND	1.85
	Clay	26.16 (±0.02)	30.09 (±0.29)	33.05 (±0.75)	ND	ND	1.95
	Potting soil	28.73 (±0.05)	31.37 (±0.02)	32.00 (±0.32)	ND	ND	4.07
Purgene	Silt loam	28.44 (±0.66)	29.91 (±0.41)	32.82 (±0.76)	ND	ND	2.86
	Clay	29.05 (±0.13)	31.71(±0.38)	ND	ND	ND	2.38
	Potting soil	23.63 (±0.04)	27.47 (±0.07)	30.02 (±0.18)	32.53 (±0.18)	ND	2.19
QIAamp Stool	Silt loam	23.09 (±0.18)	26.98 (±0.22)	30.65 (±0.21)	32.94 (±0.45)	ND	2.00
	Clay	24.91 (±0.05)	28.45 (±0.13)	31.98 (±0.21)	33.19(±0.45)	ND	2.25
	Potting soil	24.73 (±0.05)[c]	27.96 (±0.11)[c]	30.37 (±0.32)	32.99(±1.10)	ND	2.33
Epicentre	Silt loam	24.26 (±0.04)	25.69 (±0.03)	29.01 (±0.16)	32.12 (±0.26)	34.08 (±1.12)	2.40
	Clay	23.27 (±0.03)	26.65 (±0.05)	30.27 (±0.24)	33.73 (±0.25)	ND	1.93
	Potting soil	23.88 (±0.03)	27.13 (±0.15)	30.59 (±0.22)	32.08 (±1.08)	ND	2.27
Ultraclean™	Silt loam	21.83 (±0.10)	24.64 (±0.06)	30.29 (±1.43)	32.90 (±0.51)	34.10 (±0.19)	2.02
	Clay	21.27 (±0.17)	24.54 (±0.09)	28.37 (±0.14)	31.62 (±0.01)	34.30 (±0.43)	2.01
	Potting soil	23.20 (±0.11)	26.17 (±0.09)	29.36 (±0.19)	31.74 (±0.54)	33.82 (±0.73)	2.36

[a]PCR efficiencies were calculated using the formula, $E = 10^{(-1/\text{slope})}$.
[b]ND, not determined. Average Cp was only calculated for those samples in which three out of three replicates produced a positive result.
[c]Sample was positive for the inhibition assay and required a 1:2 dilution in order to obtain a positive PCR result.

Table 2
Limit of detection (LOD) of each extraction method for three soil types using the *F. tularensis fopA* real-time PCR assay on the Roche LightCycler®

Extraction method	LOD (CFU/g soil)		
	Silt loam	Clay	Potting soil
PowerMax™	100	100	100
UltraClean™	20	20	20
Epicentre	100	1000	1000
Purgene	2000	20,000	200
QIAamp Stool	500	500	500

F. tularensis DNA in all soil types tested. On the other hand, the UltraClean™ kit required the least amount of time to complete, and its cost was reasonable ($3.30 per sample) and comparable to both the QIAamp and Epicentre methods.

All of the protocols were easy to perform and not technically challenging or demanding. None of the kits required the use of expensive or unusual chemicals or reagents not supplied by the manufacturer. However, both kits from MoBio (UltraClean™ and PowerMax™) required the use of special vortex adapters, which can be purchased from Mo-Bio for approximately $65.00 each.

4. Discussion

Because of increasing concerns about bioterrorism, there is a renewed interest in *F. tularensis* [3,27]. Given the importance of characterizing the environmental background of *F. tularensis* and the recent molecular detection of *Francisella* species from soil [13,14], we felt it necessary to systematically evaluate several commercial DNA extraction methods for their ability to extract *F. tularensis* DNA from soil samples before embarking on future large-scale environmental surveys for potential reservoirs of *Francisella* species. Therefore, the aim of this study was to compare various commercial DNA extraction kits for *F. tularensis* DNA isolation from spiked soil samples. We evaluated the purity of extracted DNA using a real-time PCR assay targeting the *F. tularensis fopA* gene [25] and determined the presence of PCR inhibitors by using a PCR inhibition assay previously developed at USAMRIID [26]. Because *Taq* polymerase is highly sensitive to humic acid and other potential inhibitors found in soil and PCR amplification is a major use of extracted soil DNA, we chose to use PCR-based methods in evaluating the extracted DNA's purity, as was done in other studies [19,22]. Unlike other studies, however, we used real-time PCR assays on the Roche LightCycler®, which have a higher sensitivity than standard PCR using gel-based detection and are more amendable to high-throughput screening of soil samples.

Because soil types can vary greatly in their physical and chemical properties, and it has been shown that soils high in clay or organic matter pose particular challenges to obtaining high-quality DNA [22], we chose to evaluate three soil types (silt loam, clay, and commercial potting soil) with a wide range of organic matter (1.8–64.2%) and moisture content (6.3–69.2%). Soils are a particularly challenging matrix because extracting DNA often results in co-extraction of humic acids and other substances that inhibit *Taq* polymerase [19,28]. Therefore, we evaluated each method's ability to remove PCR inhibitors by using an internal positive control Taqman-based assay. In particular, this assay has been shown to be sensitive to a variety of potential PCR inhibitors found in the soil including fulvic acid and soil, peat, and leonardite humic acids [26]. All of the methods tested were able to remove PCR inhibitors from the silt loam and clay soil types, but one method (QIAamp) was not able to completely remove PCR inhibitors from the potting soil (3 out of 6 samples showed some level of inhibition). Humic acids are composed of higher molecular weight materials containing nitrogen in cyclic forms and aromatic rings formed by polycondensation during the decomposition of organic matter in soil [29]. Therefore, it is not surprising that potting soil, which has very high levels of organic matter (64.2%), would pose a particular challenge for PCR. Fortunately, most soils obtained from the natural environment do not contain these high levels of organic matter and will likely approximate the levels seen in clay or silt loam (i.e., about 2%).

We chose to use γ-irradiated *F. tularensis* in this study based on previous work showing no difference in the LOD of both live and γ-irradiated *F. tularensis* (LOD of 10 CFU/ml diluent for both) extracted using the IsoCode Stix DNA Isolation Device from Schleicher & Schuell (Keene, N.H.) [30]. In our study, we showed that the detection limit of *F. tularensis* varied between 20 and 20,000 CFU/g soil, depending on the soil type and the extraction method. For DNA obtained from a pure culture of *F. tularensis*, we determined the LOD of the real-time *fopA* assay to be 10 CFU. PCR efficiencies for the dilution series of spiked *F. tularensis* into each soil type for each extraction kit were determined and presented in Table 1. While the PCR efficiency of the *fopA* assay using DNA standards obtained from a pure culture of *F. tularensis* was 1.99, many of the PCR efficiencies of the *F. tularensis*-spiked soil samples were greater than the optimal efficiency of 2.00, presumably due to matrix effects. Our results showed that the UltraClean™ kit could detect *F. tularensis* from all soil types tested at a limit of detection of 20 CFU/g soil, which closely approximates the LOD obtained from pure culture. The LOD obtained from the PowerMax™ was slightly higher (100 CFU/g soil) in all three soil types. It is not surprising that the two kits from MoBio outperformed the others since they are designed and marketed specifically for extracting DNA from soils. In addition, since the MoBio methods include mechanical lysis by bead beating, our results support the results of previous studies [19,31–34] in which higher DNA yields were obtained with bead mill homogenization.

The goal of this study was to compare the performance of five commercially available DNA extraction methods for their ability to recover *F. tularensis* DNA from spiked soil samples of varying composition. Our results demonstrate that the PowerMax™ and UltraClean™ kits were the most sensitive methods for extracting *F. tularensis* DNA in soil samples as evaluated using a real-time *F. tularensis fopA* PCR assay. Using these sensitive DNA extraction methods, coupled with PCR, future studies can be performed that will hopefully shed light on the natural reservoirs of *F. tularensis* in the environment and potentially uncover further biodiversity of this important group of microorganisms.

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The views, opinions, and findings contained herein are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

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